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Prenatal Triclosan Exposure and Anthropometric Measures including Anogenital Distance in Danish Infants

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Abstract

Background: Triclosan (TCS) is widely used as an antibacterial agent in consumer products such as hand soap and toothpaste and human exposure is widespread. TCS is suspected of having endocrine disrupting properties, but few human studies have examined the developmental effects of prenatal TCS exposure.

Objectives: To prospectively examine associations between prenatal TCS exposure and anthropometric measures at birth and anogenital distance (AGD) at three months of age.

Methods: Pregnant women from the Odense Child Cohort (n=514) provided urine samples around gestational week 28 (median 28.7 weeks, range 26.4 – 34.0) and urinary TCS concentration was measured by LC-MS/MS. Multiple linear regression analysis was used to examine associations between prenatal TCS exposure and measures of size at birth (birth weight, length, head and abdominal circumference) and AGD at three months of age (median 3.3 months, range 2.3 to 6.7 months) controlling for potential confounders.

Results: Newborn boys in the highest quartile of prenatal TCS exposure had a 0.7 cm (95% CI: -1.2, -0.1, p=0.01) smaller head circumference compared with boys in the lowest quartile. Additionally in boys, inverse associations of borderline statistical significance between prenatal TCS exposure and abdominal circumference at birth and AGD at three months were observed (p-values <0.10). Prenatal TCS exposure was not significantly associated with any of the outcomes in girls. However, fewer girls had AGD measured and we observed no significant interactions between child sex and prenatal TCS-exposure in anthropometric measures at birth.

Conclusion: Prenatal TCS-exposure was associated with reduced head and abdominal circumference at birth and reduced AGD at three months of age in boys, although the two latter findings were statistically non-significant. These findings require replication, but are compatible with an anti-androgenic effect of prenatal TCS exposure on fetal growth in boys.

Introduction

Triclosan (TCS) is a biocide used as an antibacterial and antifungal agent in a number of consumer products such as toothpaste, mouthwash, disinfectants and soaps (Dann and Hontela 2011). Evidence from *in vitro* and animal studies suggest endocrine disrupting properties of TCS including anti-androgenic activity and disturbance of thyroid hormone action (Ahn et al. 2008; Axelstad et al. 2013; Gee et al. 2008; Kumar et al. 2009; Paul et al. 2010; Paul et al. 2012; Veldhoen et al. 2006). Human exposure to TCS is widespread, and studies in pregnant women have observed detectable levels of TCS in the vast majority of the women (Casas et al. 2011; Philippat et al. 2014; Wolff et al. 2008). Moreover, TCS has been detected in amniotic fluid, indicating that TCS can enter the fetal environment through placental transfer (Philippat et al. 2013). Fetal life is considered a particularly vulnerable period for exposure to endocrine disrupting chemicals, as hormonal disturbances during organ development may introduce irreversible changes (Drake et al. 2009; MacLeod et al. 2010; van den Driesche et al. 2011; Welsh et al. 2008). However, little is known about the potential adverse effects of human environmental exposure to TCS during fetal life (Wolff et al. 2008; Philippat et al. 2012; Philippat et al. 2014). A statistically non-significant inverse association between boys prenatally exposed to TCS and birth length was reported in a cohort study from the US (Wolff et al. 2008). In a French study among 520 male newborns, prenatal triclosan exposure was inversely associated with prenatal growth parameters measured by ultrasound around gestational week 33, and statistically non-significantly associated with reduced head circumference at birth (Philippat et al. 2014).

Anogenital distance (AGD), the distance from the anus to the genitals, is sexually dimorphic with males having a 50-100% longer AGD than females (Hsieh et al. 2008; Salazar-Martinez et

al. 2004; Swan et al. 2015). In rodents, the AGD has been shown to be determined by fetal androgen action during early stages of fetal development (Welsh et al. 2008). A reduced AGD in males may thus be indicative of insufficient testosterone during early stages of development of male reproductive organs, while an increased AGD in females suggests excessive androgen exposure during early stages of development of female reproductive organs (Welsh et al. 2008). In humans, a reduced AGD has been observed among boys with the genital malformations hypospadias and cryptorchidism (Hsieh et al. 2008; Hsieh et al. 2012; Jain and Singal 2013). Moreover, prenatal exposure to bisphenol A and phthalates have been associated with reduced AGD in human male infants (Miao et al. 2011; Swan et al. 2005; Bustamante-Montes et al. 2013; Suzuki et al. 2012; Swan 2008; Bornehag et al. 2014; Swan et al., 2015). Little is known about the relationship between the length of the AGD in females and female reproductive system characteristics (Mendiola et al. 2012). We have not been able to identify any human studies examining associations between prenatal exposure to TCS and AGD.

Since only very little is known about the potential effects of prenatal TCS exposure in humans, the aim of our study was therefore to examine the associations between maternal urinary excretion of TCS as a measure of prenatal TCS exposure and birth outcomes as well as measurement of AGD at three months of age stratified by child sex.

Materials and Methods

Study population

The study was based on data from Odense Child Cohort (Kyhl et al., 2015). Briefly, newly pregnant women residing in the Municipality of Odense, Denmark between January 1, 2010 and December 31, 2012 were recruited at gestational age 8 to 16 at a voluntary information meeting

about ultrasound examinations, at first antenatal midwife visit, or at the ultrasound examination at Odense University Hospital. In total, 6,707 pregnant women were eligible for the study, though only 4,017 were informed about it. As of November 2014, 2,874 women (42.9% of the total number eligible) were enrolled in the cohort (Kyhl et al., 2015).

While recruitment to the Odense Cohort was still ongoing, a subset of women with singleton pregnancies (n=565) had TCS measurements in urine samples collected around week 28 of pregnancy (median 28.7 weeks, range 26.4 – 34.0). The subset of women was selected based on availability of urine samples. The first 196 samples were selected randomly among the women enrolled in the Odense Child Cohort between September 2010 and June 2011, whereas the last 369 women were selected within the remaining women who were enrolled by January 2012, who had available information from questionnaires, urine samples, birth records and clinical examination of the child at three months of age. Of the 565 women, 51 women were excluded due to non-Caucasian origin (n=30), missing information on ethnicity (n=16), or missing data on child sex (n=5) leaving 514 mother-child pairs (273 males and 241 females) eligible for analyses.

The study was approved by the local ethical committee, and the women gave written consent to participate in the study. The research was conducted in accordance with the principles of the Declaration of Helsinki.

Birth outcomes

From birth records we obtained information about maternal pre-pregnancy BMI, gestational age (days) at birth and birth measures such as birth weight (grams), length (cm), head circumference (cm) and abdominal circumference (cm).

AGD and penile measurements

Three months after the expected date of birth, regardless of actual gestational age at birth, the children were invited to a clinical examination (median age 3.3 months, range 2.3 to 6.7 months), which included measurements of length, weight and AGD. In addition, genital malformations were noted. Two different measures of AGD were made using a Vernier caliper (Spi digiMax) in both boys and girls: In girls a short AGD was measured from center of anus to posterior fourchette (AGDaf) and a long AGD from the center of anus to the top of clitoris (AGDac). Correspondingly in boys, a short AGD was measured from the center of anus to the posterior base of scrotum (AGDas) and a long AGD was measured from the center of anus to the cephalad insertion of the penis (AGDap). Penile width was measured also using a Vernier caliper. In each child, the genital measures were repeated three times, and an arithmetic mean was calculated.

Among the 514 mother-child pairs, 252 males and 179 females had minimum one AGD-measurement and 250 males had minimum one penile measurement at the clinical examination around three months of age. AGD measurements were initiated in August 2011, but due to technical difficulties in measuring AGD in girls, these data were valid from October 2011. Data on AGD were therefore available on fewer girls than boys. Due to exclusion of children with only one or two repeated genital measures or missing data on the included covariates in the statistical analyses, n=245 (AGDas), n= 236 (AGDap), n=241 (penile width), n= 178 (AGDaf), and n= 176 (AGDac) were included in the statistical analyses.

Four technicians measured AGD in both the boys and the girls. The coefficient of variation (CV) was below 10% for all the triplicate AGD measurements, except for AGDaf, in which two girls had CVs of 0.10 and 0.14, respectively. We conducted subanalyses, in which those two girls

were excluded. Additionally, we conducted subanalyses, in which we also included the children, who only had one or two AGD-measurements (n=1 (AGDac), n=1 (AGDas), n=6 (AGDap), n=3 (penile width)). Among those with two AGD measurements the average were used as outcome measure.

TCS measurements

Urine samples from the pregnant women around week 28 of gestation were collected in the morning fasting and subsequently stored at ≤ –20 °C in freezers at the Odense Patient data Explorative Network (OPEN) until chemical analyses of total (free and conjugated) TCS by isotope dilution TurboFlow-liquid chromatography-tandem mass spectrometry (LC-MS/MS) with preceding enzymatic deconjugation (Frederiksen, Aksglæde et al. 2013). In short, the 565 samples were analyzed in 17 batches. The first 196 samples were analyzed between December 2011 and January 2012 and the following 369 samples were analyzed approximately one year later at the end of year 2012. TCS levels were similar in 2011 and 2012 measurements (p=0.44, assessed by one-way analysis of variance). Each batch included standards for calibration curves, about 35 unknown samples, two blanks, two urine pool controls and two urine pool controls spiked with TCS standards at low and high level. The inter-day variation, expressed as the relative standard deviation (RSD) was ≤14% in both spike levels. The recovery of spiked samples was >77%. We used the same control materials during both measuring periods, and there was no difference in TCS concentration in the spiked urine control material. The level of detection for TCS was 0.06 ng/mL. More details on urinary TCS excretion levels in the present cohort as well as levels of other phenols measured have recently been published in (Frederiksen et al. 2014).

Urinary osmolality, which is a measure of urinary dilution, was measured by freezing point depression method using automatic cryoscopic osmometer (Osmomat® 030 from Gonotec, Berlin, Germany). For each ninth sample measurement, a urine pool as control was measured. Mean urinary osmolality for this control pool (N=77) was 0.825 Osm/kg with a relative standard deviation (RSD) of 1.85%. The median (5^{th}, 95^{th} percentile) osmolality of all urine samples included in this study was 0.64 (0.209, 0.930) Osm/kg.

Statistics

TCS concentrations were adjusted for urinary osmolality normalized to the median osmolality of all samples (0.64 Osm/kg) to correct for dilution of the urine. This was done to all samples with a measured TCS concentration above LOD by dividing the individual urinary TCS concentration (ng/mL) with the individual osmolality of the urine sample (Osm/kg) and multiplying with the median osmolality of all samples (Osm/kg) (Lassen et al. 2013). Urinary TCS concentrations below LOD were not adjusted for osmolality, but substituted by LOD/$\sqrt{2}$. Osmolality adjusted TCS (ng/$mL_{(osm)}$) and the samples below LOD were divided into sex-specific quartiles based on the distribution among the 273 males and 241 females, respectively. The osmolality adjusted TCS concentrations and the samples below LOD were also entered in the statistical model as a continuous variable and because of skewed distribution, log2-transformed. The birth outcomes (weight, length of the child, head circumference and abdominal circumference), AGD measurements and the penile width were left untransformed due to acceptable normal distributions of the residuals.

We calculated the distribution of anthropometric measures at birth and anogenital distance as well as the correlations (Spearman correlation coefficients) between the genital measures among

the boys and girls, respectively. Differences in distributions of TCS concentrations according to population characteristics were assessed by one-way analysis of variance. Multiple linear regression analysis was used to analyze the associations between urinary TCS excretion and birth outcomes and AGD-measurements adjusted for potential confounders. We tested for linear trend across TCS quartiles in regression models by means of ordinal TCS quartiles using integer values 1 to 4. Confounders included in multivariable models were factors known *a priori* to be important predictors of birth outcomes or AGD. AGD measurements vary with age and weight of the child, and because the clinical examination was scheduled to take place three months after expected date of birth we constructed a measure of 'post-conceptional age' defined as the sum of gestational age at birth (in days) and the age of the child at the AGD measurements (in days). Analyses of associations between TCS and AGD were thus adjusted for the post-conceptional age and individual weight-for-age standard deviation score (SD-score) (Swan 2008) calculated by use of Danish longitudinal growth data (Tinggaard J et al. 2014). Additionally, to examine whether systematic differences in AGD-measurements between the examiners could confound the association between TCS and AGD as outcome, we performed sensitivity analyses with inclusion of information on the examiner as a categorical variable with five categories (four different examiners and a category for missing information of examiner).

Analyses of associations between TCS and infant size at birth (birth weight and length, head and abdominal circumference) were adjusted for parity (primiparous/multiparous), maternal smoking during pregnancy (yes/no), pre-pregnancy BMI (<20, 20-25, 25+ kg/m2) and gestational age (days). All results were presented stratified by sex given *a priori* interest in sex differences. However, we also tested for potential interaction between child sex and TCS exposure in models with birth weight and length, head and abdominal circumference as outcomes, respectively, by

inserting a product interaction term of child sex × maternal urinary TCS levels (continuous, osmolality-adjusted, log2-transformed TCS concentration) in the statistical models. Percentage change in outcomes was calculated as the difference between highest exposed group and the reference group divided by the intercept x 100, where the reference values on the covariates were gestational age at birth at 280 days, pre-pregnancy BMI between 20-25 kg/m2, primiparous and non-smokers.

There are known ethnic differences in size at birth and AGD (Papadopoulou et al, 2013; Sathyanarayana et al. 2010) and we therefore excluded non-Caucasian women to avoid confounding due to ethnicity. However, we performed sensitivity analyses in which we included the non-Caucasian women. We also performed sensitivity analyses in which maternal height and pre-pregnancy weight were included in the statistical models instead of pre-pregnancy BMI.

Residual plots were used to examine the model assumption of homogeneity of variances, whereas the normality of the distribution of residuals was examined graphically by means of histograms and normal probability plots (data not shown). Associations were considered statistically significant at the $p<0.05$ level and observations with missing data on variables included in the statistical analyses were excluded. We focused on associations, where we observed trends across quartiles, as we considered single significant observations without a dose-response trend as less reliable.

All data analysis was performed using SAS version 9.1 (SAS Institute Inc., Cary, NC, USA).

Results

The mean age of at birth among the 514 women was 31.0 years, 55% of the women were primiparous and 3.6% smoked during pregnancy. Mean gestational age at birth was 280 days

(range 205 to 296 days) in girls and 280 days in boys (range 221 to 297 days). Detectable maternal urinary TCS concentration was found in 83% of the samples (girls: 84%, boys: 82%). The median unadjusted urinary TCS concentration was 0.88 ng/mL and the 95th percentile and the maximum unadjusted TCS concentration were 428 ng/mL and 2614 ng/mL, respectively. Median (5th, 95th percentiles) osmolality adjusted TCS concentrations stratified on child sex are shown in table 1. No significant associations between maternal osmolality adjusted TCS levels and population characteristics were observed either in girls or boys, and maternal osmolality adjusted TCS levels were not significantly different between girls and boys. Data on covariates were missing on less than 2.5% of the observations. The 514 women with TCS measurements eligible for this study did not differ significantly from the other singleton birth-giving women enrolled in the Odense cohort with respect to gestational age at delivery, maternal parity and age (data not shown), whereas the mean birth weight among the children included in this study was statistically significantly higher (3554 g vs. 3495 g). There were fewer smokers in the present cohort although not statistically significant (3.6% vs. 5.1%).

Mean AGD measures and birth outcomes stratified on child sex are shown in table 2. The correlations between the two different AGD measures in boys and girls were r=0.63 (p<0.0001), and r=0.61 (p<0.0001), respectively. As expected the mean AGD measures in the boys were nearly twice the corresponding AGD measure in the girls (table 2). Penile width was weakly correlated with AGDas (r=0.22, P=0.0006) and AGDap (r=0.14, p=0.03).

Associations between maternal urinary TCS levels and size at birth

Among the boys we observed a linear dose-dependent inverse association between maternal urinary TCS levels and head circumference indicated by the trend across TCS quartiles (p-

trend=0.01); for every doubling in TCS concentration, head circumference decreased by 0.06 cm among the boys (95% CI:-0.11, -0.002, p=0.04) (Table 3 and Figure 1). When maternal urinary TCS levels was categorized in quartiles, boys in the highest TCS quartile had, on average, a 0.7 cm (95% CI: -1.2, -0.1, p=0.01) smaller head circumference compared to boys in the first quartile. This corresponds to a reduction of 2.0% (95% CI: -3.5%, -0.4%) between fourth versus first exposure quartile. Maternal urinary TCS levels was also inversely associated with abdominal circumference among the boys in a dose-dependent manner, although not statistically significantly (p-trend=0.07) (Table 3 and Figure 1). Boys in the highest TCS quartile had on average a 0.6 cm smaller abdominal circumference compared with the first TCS quartile, although the estimate did not reach statistical significance (95% CI: -1.2, 0.0, p=0.07). This corresponds to a reduction in mean abdominal circumference of 1.8% (95% CI: -3.6%, 0.1%). We observed no significant associations between prenatal TCS exposure and birth weight (e.g. for boys in the highest exposure quartile: β= -81 (95% CI -218, 56, p=0.25), and there was no consistent trend in estimated associations with increasing exposure. We observed a significant positive association between birth length and the third versus first quartile of TCS, but in the other quartiles the beta values were close to the null, without a consistent pattern according to prenatal TCS exposure (Table 3 and Figure 1).

We found no significant associations or consistent dose-response relations between prenatal TCS exposure and any of the measures of size at birth in girls (Table 3 and Figure 1).

The interaction terms between child sex and prenatal TCS exposure in models with birth weight and length, head and abdominal circumference as outcomes were not statistically significant (Table 3), but the population size (i.e. the statistical power) was probably too small for test of interaction.

Associations between maternal urinary TCS levels and AGD at three months of age

In the boys, inverse associations between maternal urinary TCS levels and both the long and the short AGD measure were observed, although the estimates did not reach statistical significance (p-values < 0.10): every doubling in TCS concentration was associated with a decrease of 0.2 mm (95% CI: -0.3, 0.0, p=0.08) in AGDas and a decrease of 0.2 mm (95% CI: -0.4, 0.0, p=0.05) in AGDap (Table 4). Penile width was not associated with maternal urinary TCS levels.

We observed no significant associations or consistent dose-response associations between prenatal TCS exposure and anogenital distance in the girls (although based on fewer numbers than in the boys) (Table 4).

Sub-analyses showed that inclusion of children with only one or two of the three AGD-measurements did not appreciably change the results (less than 10% change in estimates) (results not shown). Neither did inclusion of examiner information, inclusion of non-Caucasian women or inclusion of maternal height and pre-pregnancy weight instead of pre-pregnancy BMI in the analyses substantially change the observed associations (results not shown).

Discussion

In this prospective study we observed an inverse association between prenatal TCS exposure measured by maternal urinary TCS excretion and head circumference in newborn boys. There were no clear associations between prenatal TCS exposure and birth weight or length in boys. We also observed reduced abdominal circumference and shorter AGD in the boys, although these estimates did not reach statistical significance (p<0.10). In contrast, we observed no significant associations between prenatal TCS exposure and any of the outcomes in girls. However fewer girls had AGD measured. Analyses of interactions between sex and prenatal TCS

exposure were not statistically significant, which was probably due to low statistical power to perform interaction analyses, because of small sample size.

The observed reduction of 0.7 cm in head circumference between highest versus lowest exposed quartile in boys was of similar magnitude as differences in head circumference between boys and girls observed in this study (mean difference = 0.6 cm) and reported at birth in other studies (Greil 2006; Sankilampi et al. 2013). For example in a Finnish register-based study based on 533,666 singletons born 1996-2008 girls born at 40 weeks of gestation had on average a 0.65 cm smaller head circumference than boys (Sankilampi et al. 2013).

Few human studies have examined prenatal TCS exposure and birth outcomes and to our knowledge no human study has examined associations with AGD. Consistent with the present study, a French mother-child cohort study including 520 newborn boys observed a statistically non-significant inverse association between prenatal triclosan exposure and head circumference, but no associations with length or weight at birth were observed (Philippat et al. 2014). In a cohort study from the US including 339 mothers and their offspring, Wolff et al. (2008) reported a statistically non-significant inverse association between prenatal TCS exposure and birth length among boys. Consistent with the present study, Wolff et al. did not observe any associations between prenatal TCS exposure and birth outcomes among girls (Wolff et al. 2008). It is noteworthy that both earlier studies reported considerable higher median maternal urinary TCS concentrations (Wolff et al. 2008: 11 ng/mL, Philippat et al. 2014: 30 ng/mL) than our study (0.88 ng/mL). However, the variation in TCS levels in the present study was large (5^{th}, 95^{th} percentile and maximum: <LOD, 428 and 2614 ng/mL, respectively), suggesting that some Danish pregnant women were as high exposed as women in previous studies (Wolff et al. 2008; maximum TCS concentration: 1790 ng/mL) (Phillipat et al. 2014; 95^{th} percentile TCS

concentration: 755 ng/mL). The differences in overall urinary TCS excretion across studies may reflect differences in consumer behavior between countries.

Studies in rats have not observed effects of prenatal TCS exposure on size at birth or AGD (Axelstad et al. 2013; Rodriguez and Sanchez 2010). However, reproductive effects, including reduced levels of follicle-stimulating hormone (FSH), luteinizing hormone (LH) and testosterone have also been shown in adult male rats exposed to 20 mg/kg bw/day TCS during a period of 60 days (Kumar et al. 2009) and reduced sperm production in rats exposed to 50 and 200 mg/kg TCS for 8 weeks (Lan et al. 2013). This suggests that the hypothalamic-pituitary-gonadal axis may be affected by TCS exposure. Furthermore, in a number of rat studies, TCS exposure has consistently been shown to induce hypothyroxinemia in dams exposed during gestation and lactation (Paul et al. 2010; Paul et al. 2012; Axelstad et al. 2013), as well as in neonate pups exposed prenatally (Paul et al. 2010; Paul et al. 2012). This suggests that TCS may have thyroid hormone disrupting effects. Additionally, in vitro studies have suggested different modes of action of TCS, including estrogenic, androgenic, anti-androgenic and gestagenic effects (Ahn et al. 2008; Gee et al. 2008; Christen et al. 2010; Schiffer et al. 2014).

This study was not designed to elucidate mechanisms of action. However, because we observed associations between prenatal TCS exposure and measures of growth only among the boys (although interaction analyses were non-significant), and the difference in mean head circumference between boys with the highest versus lowest TCS exposure was comparable to the average difference in head circumference between boys and girls reported for other populations (Sankilampi et al. 2013), we speculate that our findings may be compatible with an anti-androgenic mechanism of action of TCS. Nevertheless, other mechanisms of action of TCS leading to diminished growth are also possible, for instance, such as a perturbation of the thyroid

axis. These results need to be confirmed in future studies, ideally with measurement of hormone levels. A causal relationship between prenatal TCS exposure and reduced head circumference, would be of considerable public health significance as reduced head circumference at birth has been associated with impaired cognitive performance (Lundgren et al. 2001; Veena et al. 2010).

The AGD in males may be a marker of testicular dysgenesis syndrome (TDS) (Thankamony et al. 2014; Juul et al. 2014) suggesting that some male reproductive disorders may be symptoms of a common underlying fetal testicular dysgenesis, which may be caused by a disturbance in the Sertoli cell and Leydig cell differentiation during fetal life leading to impaired testosterone production (Skakkebæk et al. 2001). Based on results from animal studies, shortening of the AGD in newborn males is attributed to impaired androgen action during a particularly sensitive developmental window, which is believed to correspond to gestational week 8 to 14 in humans (Welsh et al. 2008). The observation in this study of reduced AGD in boys, although statistically non-significant, is therefore consistent with an anti-androgenic effect of TCS on the fetus. This is also in accordance with the in vitro studies showing anti-androgen action of TCS (Ahn et al. 2008; Gee et al. 2008), although a study examining developmental effects following *in utero* TCS exposure in rats has not observed significant changes in AGD in male offspring (Axelstad et al. 2013) and no association was observed between prenatal triclosan exposure and undescended testis in a rather small study of 151 male newborns (Chevrier et al. 2012).

TCS is quickly metabolized with a urinary excretion half-life of less than 24 hours (Sandborgh-Englund et al. 2006). A single spot urine sample collected around gestational week 28 may therefore potentially fail to accurately reflect average fetal exposure during the entire pregnancy or during the developmental window early in fetal life suggested to be particularly sensitive for the formation of the AGD (Welsh et al. 2008). However, studies of temporal variability in

urinary excretion of TCS have indicated reasonable temporal consistency in TCS excretion (Meeker et al. 2013; Philippat et al. 2013; Lassen et al. 2013), with interclass correlation coefficients among pregnant women between 0.47 and 0.58 (Meeker et al. 2013; Philippat et al. 2013) suggesting relatively stable exposure sources to TCS throughout gestation, although some exposure misclassification is to be expected when using a single urine sample collected during pregnancy for exposure classification. This may lead to underestimation of an association between prenatal TCS exposure and AGD. The third trimester of pregnancy is characterized by rapid fetal weight gain and urine samples collected around 28 week of gestation may therefore characterize a relevant window of exposure for fetal growth effects. In this study AGD was measured at around 3 months of age. In a study by Thankamony et al. (2009) AGD-measurements at three months of age have been shown to be weakly, though significantly correlated with AGD-measurements at birth in both boys (r=0.30) and girls (r=0.26).

In this study a relatively large number of statistical analyses were carried out, and some of our results may therefore be chance findings due to multiple testing. We had relatively limited sample size and therefore limited statistical power for analyses of sex-specific associations between TCS exposure and growth measures. The suggested sex-specific differences in outcomes are therefore hypothetical and should be confirmed in other studies.

Theoretically, selection bias may have affected the results. Only 43% of the eligible women participated and participants were more often of Danish origin and were better educated than non-participants. The women included in this study had a lower prevalence of smoking during pregnancy (~4%) compared with non-participants (12%), who were women giving birth between 2010 and 2013 and living in the recruitment area of the Odense Child Cohort (Kyhl et al., 2015). The mean age at delivery in our study was, however, identical with the mean age of all Danish

women giving birth in Denmark in 2011 (Danish Health and Medicines Authority 2012), and the median TCS concentration among the pregnant women in this study was comparable with levels observed in Danish children aged 6-11 years and their mothers in samples collected in 2011 (median 0.46 ng/mL and 0.64 ng/mL, respectively) (Frederiksen, Nielsen et al., 2013). The women in this study had no prior knowledge of their TCS exposure, which therefore is unlikely to have affected their willingness to participate. Although all estimates of associations were adjusted for relevant confounders we cannot exclude the possibility of confounding by other factors associated with TCS and growth measures, e.g. co-exposure to other environmental chemicals or lifestyle factors. Our findings therefore need to be confirmed in other human studies.

Conclusions

Prenatal TCS exposure was associated with reduced head and abdominal circumference at birth and a reduced AGD at three months of age in boys, although the two latter findings did not reach statistical significance ($p<0.10$). No associations between prenatal TCS exposure and birth outcomes and AGD were found in girls, although fewer girls had AGD measured and the interactions between child sex and TCS were not statistically significant. Due to the suggestive sex differences we speculate that our findings are compatible with an anti-androgenic effect of prenatal TCS exposure on fetal development in boys. However, our findings need to be confirmed in other populations and biological mechanisms should be elucidated.

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Table 1 Population characteristics according to median (5^{th}, 95^{th} percentile) maternal urinary TCS excretion ($ng/mL_{(osm)}$) stratified by child sex among 514 mother-child pairs.

		Girls		**Boys**
Population characteristics	*n*[a] (%)	Median TCS (5^{th}, 95^{th} percentile) $ng/mL_{(osm)}$	*n*[b] (%)	Median TCS (5^{th}, 95^{th} percentile) $ng/mL_{(osm)}$
All	241 (100)	1.01 (<LOD, 536)	273 (100)	0.96 (<LOD, 335)
Maternal age at birth				
<29 years	77 (32)	0.94 (<LOD, 536)	102 (38)	0.76 (<LOD, 210)
29 - 33 years	82 (34)	0.91 (<LOD, 474)	81 (30)	0.89 (<LOD, 160)
>33 years	81 (34)	1.43 (<LOD, 562)	84 (31)	1.14 (<LOD, 669)
Prepregnancy BMI (kg/m^2)				
<20	22 (9)	1.14 (< LOD, 20)	26 (10)	1.31 (<LOD, 538)
20-25	130 (54)	0.95 (< LOD, 503)	141 (53)	0.93 (<LOD, 182)
25+	88 (37)	1.07 (< LOD, 719)	100 (37)	0.91 (<LOD, 318)
Parity				
Primiparous	124 (51)	1.06 (< LOD, 664)	156 (57)	0.98 (<LOD, 227)
Multiparous	117 (49)	0.95 (< LOD, 503)	116 (43)	0.88 (<LOD, 538)
Maternal smoking during pregnancy				
Yes	9 (4)	0.94 (< LOD, 503)	9 (3)	0.31 (<LOD, 1.70)
No	231 (96)	1.06 (< LOD, 546)	258 (97)	0.97 (<LOD, 339)
Preterm birth[c]				
Yes	8 (3)	1.04 (0.09, 664)	9 (3)	0.82 (<LOD, 23)
No	232 (97)	1.03 (<LOD, 536)	258 (97)	0.95 (<LOD, 339)

[a]Due to missing data, the numbers do not add up to 241 for the covariates.

[b]Due to missing data, the numbers do not add up to 273 for the covariates.

[c]Birth before gestational week 37

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Table 2 Distribution (mean, ± standard deviation (SD)) of anthropometric measures at birth and anogenital distance at around 3 months of age among boys and girls from the Odense Child Cohort.

Anthropometric and AGD measures	**Girls**			**Boys**		
	n	**Mean**	**SD**	***n***	**Mean**	**SD**
Birth weight (g)	240	3502	503	267	3600	536
Birth length (cm)	239	51.8	2.2	264	52.4	2.4
Head circumference (cm)	238	34.9	1.7	264	35.5	1.8
Abdominal circumference (cm)	237	33.5	2.3	261	33.7	2.1
AGD, short (mm)	179	20.1	3.6	251	36.8	5.5
AGD, long (mm)	177	37.7	4.4	242	70.7	6.5
Penile width (mm)	-	-	-	247	13.8	1.2

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Table 3 Associations between maternal pregnancy TCS levels and birth outcomes among newborn boys and girls from the Odense Child Cohort.

	Birth weight (g)			Birth length (cm)			Head circumference (cm)			Abdominal circumference (cm)		
TCS	N	β (95% CI)	*P*-value	N	β (95% CI)	*P*-value	N	β (95% CI)	*P*-value	N	β (95% CI)	*P*-value
Boys												
TCS quartile[a]												
1st	69	Reference		69	Reference		69	Reference		68	Reference	
2nd	66	-62 (-199, 74)	0.37	66	0.1 (-0.5, 0.7)	0.83	66	-0.2 (-0.7, 0.4)	0.52	64	-0.3 (-0.9, 0.3)	0.33
3rd	66	29 (-108, 166)	0.68	65	0.7 (0.1, 1.3)	0.03	64	-0.3 (-0.9, 0.2)	0.24	64	-0.4 (-1.0, 0.2)	0.22
4th	66	-81 (-218, 56)	0.25	64	0.1 (-0.5, 0.8)	0.65	65	-0.7 (-1.2, -0.1)	0.01	65	-0.6 (-1.2, 0.0)	0.07
P-Trend[b]			0.49			0.29			0.01			0.07
Continuous[c]	267	-5.2 (-18.5, 8.2)	0.45	264	0.01 (-0.05, 0.07)	0.70	264	-0.06 (-0.11, -0.002)	0.04	261	-0.05 (-0.11, 0.01)	0.09
Girls												
TCS quartile[a]												
1st	60	Reference		59	Reference		59	Reference		59		
2nd	60	50 (-90, 190)	0.48	60	-0.5 (-1.1, 0.1)	0.11	60	-0.4 (-0.9, 0.2)	0.19	60	0.3 (-0.4, 0.9)	0.47
3rd	60	25 (-116, 167)	0.72	60	-0.4 (-1.0, 0.2)	0.24	60	-0.3 (-0.8, 0.2)	0.28	59	0.1 (-0.6, 0.8)	0.84
4th	60	36 (-104, 176)	0.62	60	-0.2 (-0.8, 0.4)	0.53	59	-0.2 (-0.7, 0.3)	0.49	59	-0.03 (-0.7, 0.6)	0.93
P-Trend[b]			0.71			0.66			0.56			0.81
Continuous[c]	240	4.7 (-7.3, 17.0)	0.43	239	-0.004 (-0.06, 0.05)	0.89	238	-0.01 (-0.05, 0.04)	0.70	237	0.00 (-0.06, 0.06)	0.998
TCS x Gender												
P-interaction[d]	507		0.39	503		0.57	502		0.24	498		0.27

All estimates are adjusted for gestational age, maternal smoking, parity and pre-pregnancy BMI. [a]Median (range) for osmolality-adjusted TCS quartiles (ng/mL$_{osm}$) in boys: 1st = <LOD (<LOD – <0.24), 2nd = 0.53 (0.24 – <0.97), 3rd = 1.69 (0.97 – <3.24), 4th = 17.8 (3.24 – 1702); in girls: 1st = <LOD (<LOD – <0.247), 2nd = 0.50 (0.247 – <1.05), 3rd = 2.03 (1.05 – <4.21), 4th = 103.1 (4.21 – 2350). [b]*P*-value for trend across TCS quartiles. [c]Log$_2$ transformed TCS concentration. [d]*P*-value for interaction (child sex × maternal urinary TCS levels (continuous log2-transformed TCS concentration)). Median gestational week of maternal urine collection in girls was 28.7 (range 27.9 to 34.0) weeks and in boys 28.7 (26.4 to 30.4) weeks.

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Table 4 Associations between maternal pregnancy TCS levels and AGDas (short AGD measure), AGDap (long AGD measure) and penile width in boys and AGDaf (short AGD measure) and AGDac (long AGD measure) in girls at three months of age from the Odense Child Cohort.

	Boys									Girls					
		AGDas (mm)			AGDap (mm)			Penile width (mm)			AGDaf (mm)			AGDac (mm)	
TCS quartile[a]	**N**	β (95% CI)	p-value	N	β (95% CI)	p-value	N	β (95% CI)	p-value	N	β (95% CI)	p-value	N	β (95% CI)	p-value
1st	64	Reference		63	Reference		63	Reference		51	Reference		50	Reference	
2nd	61	-0.9 (-2.8, 0.9)	0.33	60	0.3 (-1.7, 2.3)	0.77	59	0.0 (-0.4, 0.5)	0.81	40	-0.5 (-2.0, 1.0)	0.51	40	-0.6 (-2.4, 1.2)	0.54
3rd	62	-2.3 (-4.1, -0.4)	0.02	59	-1.2 (-3.3, 0.8)	0.24	62	-0.1 (-0.5, 0.3)	0.55	43	-0.4 (-1.8, 1.1)	0.59	43	0.1 (-1.6, 1.9)	0.87
4th	58	-1.3 (-3.1, 0.6)	0.19	54	-1.3 (-3.4, 0.8)	0.22	57	-0.2 (-0.6, 0.3)	0.44	44	-0.3 (-1.8, 1.1)	0.66	43	0.1 (-1.7, 1.8)	0.95
P-Trend[b]			0.08			0.11			0.32			0.69			0.79
Continuous[c]	245	-0.16 (-0.34, 0.02)	0.07	236	-0.20 (-0.39, 0.00)	0.05	241	-0.02 (-0.06, 0.02)	0.27	178	-0.02 (-0.15, 0.10)	0.72	176	0.01 (-0.15, 0.17)	0.89

All estimates are adjusted for weight for age standard deviation score and post-conceptional age (gestational age at birth + age of the child at the AGD measurements, days). [a]Median (range) for osmolality-adjusted TCS quartiles (ng/mL$_{osm}$) in boys: 1st = <LOD (<LOD – <0.24), 2nd = 0.53 (0.24 – <0.97), 3rd = 1.69 (0.97 – <3.24), 4th = 17.8 (3.24 – 1702); in girls: 1st = <LOD (<LOD – <0.247), 2nd = 0.50 (0.247 – <1.05), 3rd = 2.03 (1.05 – <4.21), 4th = 103.1 (4.21 – 2350). [b]*P*-value for trend across TCS quartiles. [c]Log2 transformed TCS concentration. Median age at AGD measurements in boys; 3.3 months (range 2.3 to 6.2 months), in girls; 3.3 months (range 2.3 to 6.7 months).

Figure legend

Figure 1: Differences (β-coefficients from multiple linear regression) and 95% CIs in anthropometric measures at birth among newborn boys and girls from the Odense Child Cohort in relation to prenatal triclosan (TCS) exposure quartiles (ng/mL$_{osm}$). All estimates are adjusted for gestational age, maternal smoking, parity and pre-pregnancy BMI. Median (range) for osmolality-adjusted TCS quartiles (ng/mL$_{osm}$) in boys: 1st = <LOD (<LOD – <0.24), 2nd = 0.53 (0.24 – <0.97), 3rd = 1.69 (0.97 – <3.24), 4th = 17.8 (3.24 – 1702); in girls: 1st = <LOD (<LOD – <0.247), 2nd = 0.50 (0.247 – <1.05), 3rd = 2.03 (1.05 – <4.21), 4th = 103.1 (4.21 – 2350). P-trend: *P*-value for trend across TCS quartiles.

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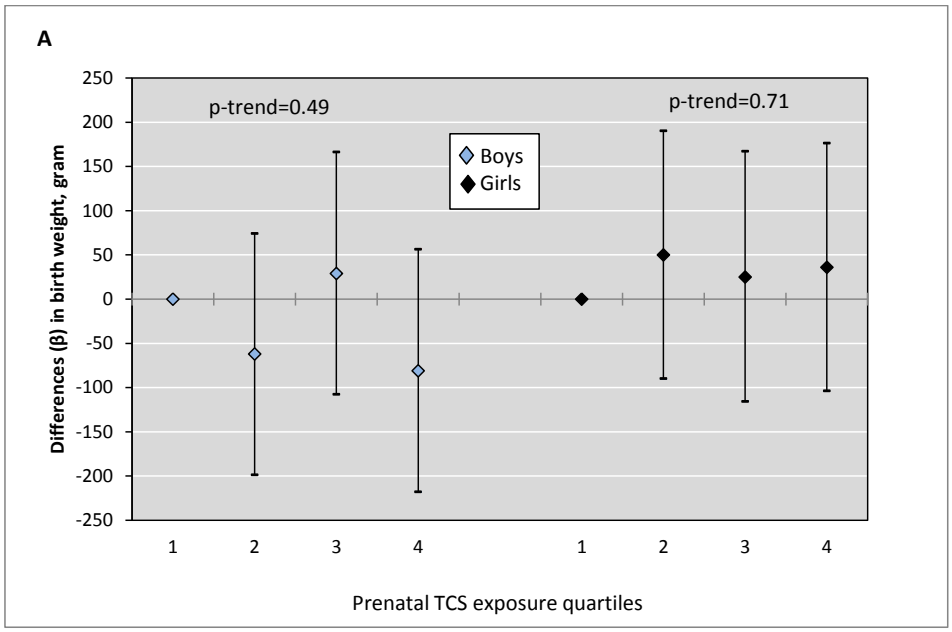
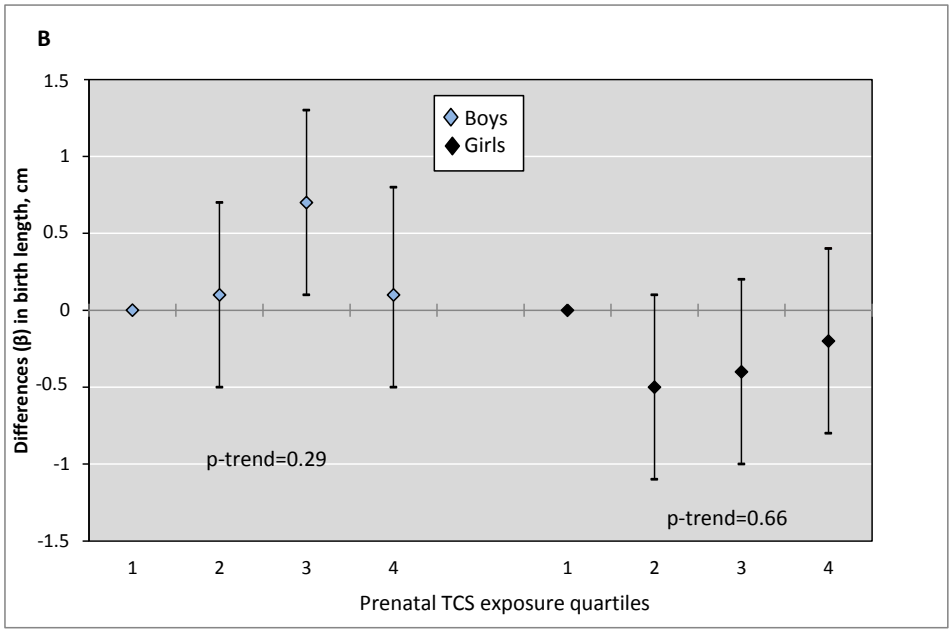
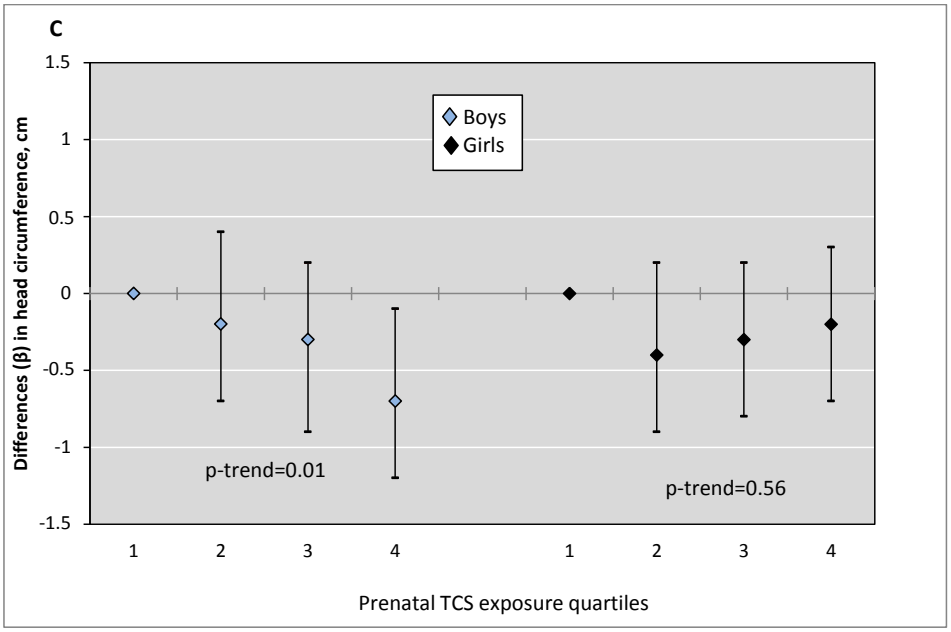
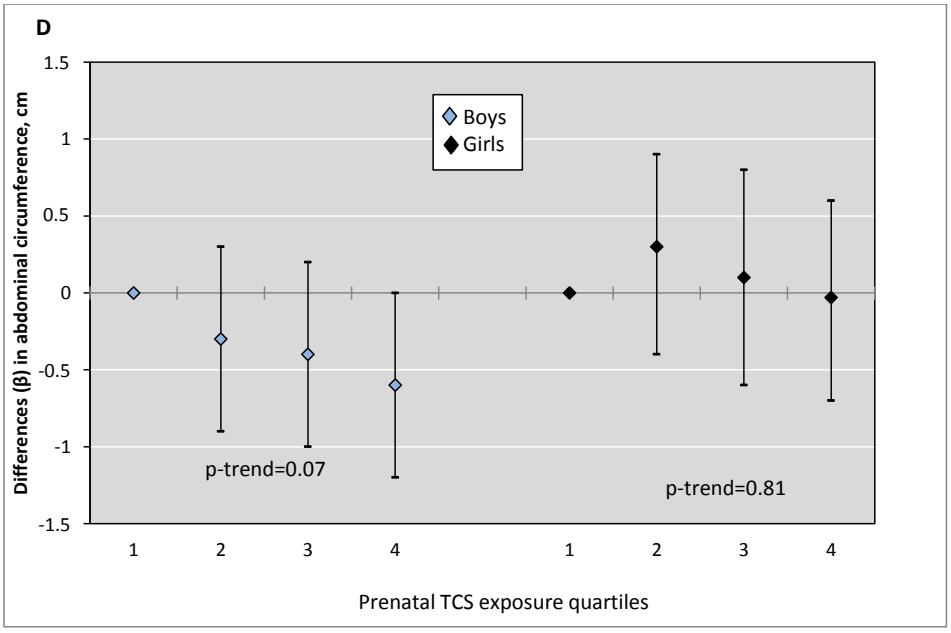
Figure 1.






C
Differences (β) in head circumference, cm
1.5
1
0.5
0
-0.5
-1
-1.5
Boys
Girls
p-trend=0.01
p-trend=0.56
1
2
3
4
1
2
3
4
Prenatal TCS exposure quartiles


D
Differences (β) in abdominal circumference, cm
1.5
1
0.5
0
-0.5
-1
-1.5
Boys
Girls
p-trend=0.07
p-trend=0.81
1
2
3
4
1
2
3
4
Prenatal TCS exposure quartiles